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Research Article

From Asia through America to Europe: Molecular and morphological evidence for a non-indigenous diatom species, *Discostella asterocostata* (B.Q.Lin, S.Q.Xie & S.X.Cai) Houk & Klee in Hungary and Croatia

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Abstract

This study presents the first occurrences of the diatom species *Discostella asterocostata* in Europe's second largest river, the Danube River and the earliest occurrence in European freshwater ecosystems. Samples collected between 2016 and 2022 in Croatia and Hungary were analysed using light and scanning electron microscopy (SEM) along with molecular techniques to confirm the presence of the species. Morphometric comparisons with known populations from Asia and the USA revealed no significant morphological differences. Molecular analyses facilitated the detection of *D. asterocostata* even in samples with low abundance and confirmed the genetic similarity with already sequenced populations.

Environmental correlations showed that *D. asterocostata* had a significant positive correlation with temperature and a negative correlation with total organic carbon and nitrogen forms, suggesting that it favours warmer waters with lower nutrient forms. Given the ongoing increase in water temperatures due to climate change, the occurrence and potential spread of this species in European rivers may be related to changing environmental conditions.

Our results emphasise the importance of integrating traditional and molecular approaches for the early detection of non-indigenous diatom species. The establishment of *D. asterocostata* in Europe raises questions regarding its ecological role and long-term impact on native phytoplankton communities and emphasises the need for continuous monitoring and research on the responses of large river ecosystems to global environmental change.

Key words: centric diatoms, ecological monitoring, JDS4, large rivers, metabarcoding, phytoplankton

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Introduction

The fundamental elements of the aquatic food web are aquatic photoautotrophs, whose changes in abundance and community composition influence other components of the food web, which can lead to a cascading effect in aquatic ecosystems. Most large and medium-sized rivers are subject to considerable anthropogenic pressure. For example, many large rivers have been dammed by the construction of reservoirs and the cumulative effects of these dams have led to a decline in native freshwater biodiversity at the regional scale (Cantonati et al. 2020).

Euplanktonic diatoms are one of the most important groups of primary producers in large rivers and are present throughout the year. Nowadays, due to global warming, the average temperature of surface waters is increasing (Dokulil 2014), leading to changes in the community structure of native aquatic microorganisms, including diatoms (Cantonati et al. 2020). In general, the number of non-indigenous species (NIS) shows an increasing trend as the problems caused by global warming are added to direct human pressure (Simberloff et al. 2013). These species do not necessarily become invasive, but they can still affect ecosystems.

NIS algae, like invasive species, are able to quickly adapt to new environments and gain a competitive advantage over native species. A species is generally considered invasive if its proliferation causes or is likely to cause economic or ecological harm or affect the health of living organisms (ISAC 2006), meaning that it has a negative impact on native species and the ecosystem. If a species is simply introduced to a new location without spreading aggressively or causing significant ecological change, the term non-indigenous species is more appropriate.

However, in the case of diatoms, the two terms are often used almost synonymously. This is likely due to the criteria used to identify invasive diatom species, which are slightly adapted to their specific ecological context. According to Coste and Ector (2000), diatom species are considered invasive if they: (i) were not recorded in France before 1990, (ii) have shown substantial proliferation and (iii) have rapidly expanded their range in running waters. In addition to these criteria, the absence of these algae from previous European floras further supports the idea of their recent introduction to the continent. These criteria differ slightly from general definitions of biological invasion, but reflect the practical approach taken in diatom biogeography, where historical distribution data are often the most reliable basis for inference.

In both cases, the competition triggered by the presence of a new species can lead to changes in the composition of native algal communities and to a decline in biodiversity and their effects on ecosystems are often difficult to predict (Sala et al. 2000). Recently, more and more studies have emphasised the phenomenon of diatom invasion (Kilroy 2004; Kilroy et al. 2009; Reid et al. 2012; Saber et al. 2022; Hu et al. 2023), often in the context of global climate change (Spaulding et al. 2010; Duleba et al. 2014; Falasco et al. 2016; Buczkó et al. 2022).

The accelerated development of molecular methods provides a good opportunity for the rapid and early detection of newly-emerging species, even if they occur in low numbers in a particular habitat (Egan et al. 2015). It is of particular significance if the species also causes environmental problems, such as the “lake snow” phenomenon, which is caused by an invasive planktonic centric diatom. Molecular methods have made it possible to understand and monitor

the formation process, representing an important step towards the long-term management of the problem (Novis et al. 2020).

This study presents data from the Mura, Drava and Danube Rivers and their national study sites for monitoring the biological quality element phytoplankton due to the presence of *Discostella asterocostata* in the samples. We report here on the first findings of this species in Europe's second largest river, the Danube and the earliest occurrence in Europe. The species was originally described in China and later found in other Asian countries and in the U.S., but there was a gap in Europe before 2016. In this article, we present the morphological characteristics of the species in comparison to known populations from Asia and the U.S. as well as the ecological characteristics while inhabiting the potamoplankton of large rivers in the Pannonian lowlands. In addition, the ecology of the species in terms of hydromorphology and water chemistry, as well as its molecular identification, was analysed using metabarcoding techniques.

Materials and methods

Study area

Croatia is divided into two ecoregions: Pannonian and Dinaric. Four very large rivers, each with a catchment area of more than 10,000 km^2, flow only in the Pannonian Ecoregion and are part of the Black Sea watershed. The Mura and Drava Rivers flow along the northern border with Hungary and the Danube on the eastern border with the Republic of Serbia. The Mura, Drava and Danube Rivers converge on Croatian territory. All three rivers have impoundments (hydroelectric dams) in their watercourses and parts with stagnant or slow-flowing water, and during flood periods, they are connected to backwaters that are potential inocula for phytoplankton species. The Sava River flows along the southern border with Bosnia and Herzegovina and flows into the Danube in Serbia. In accordance with the Water Framework Directive (European Commission 2000), biomonitoring of the above-mentioned large rivers was introduced in 2010.

The characteristics of the Croatian study sites included in this study are listed in Stanković et al. (2012), while the Hungarian ones can be found in Ács et al. (2025). All sampling sites are shown in Fig. 1.

Sampling

Water sampling for physicochemical analyses and phytoplankton sampling was carried out in Croatia in 2016, 2017 and 2019 from April to September. The Goričan sampling site on the Mura River and the sampling sites on the Drava River (Botovo and Terezino Polje) were sampled in 2016 and 2017, while all other sampling sites on the Drava River (Donji Miholjac, Belišće and 5 km above the confluence) and on the Danube River (Batina at rkm 1434 and Ilok at rkm 1300) were additionally sampled in 2019. The surface water samples were taken from the bridge or from the boat in the middle of the river, the thalweg (CEN 2015b) and used for the quantitative analysis of phytoplankton and the physical and chemical properties of the water.

Figure 1. Study area with sampling sites in Croatia and Hungary.

In the case of the Hungarian samples, monthly phytoplankton samples were taken from the Hungarian section of the Danube (at 13 sampling sites from the main arm and one site on the Mosoni Danube and one site on the Ráckevei-Soroksári Danube (RSD), which are side arms of the river) between May 2021 and April 2022. Each sample had a volume of three litres and was taken from the thalweg, 10 cm below the water surface (Kiss et al. 1996). They were stored between 4 and 8 °C until processing. In the laboratory, after shaking, the sample was filtered through a 3 µm Isopore™ polycarbonate membrane filter. The algal cells remaining on the filter membrane were re-suspended with a few millilitres of filtered water and preserved with a final concentration of > 70% a.r. absolute ethanol for subsequent DNA extraction. In addition, 1 ml of the sediment re-suspended from the filter was fixed with formaldehyde (final concentration 4%) for scanning electron microscopy of the samples from March, May and July.

Environmental variables

For the physical and chemical analyses of the Croatian water, the water temperature was measured on site using a digital thermometer. Conductivity and pH were measured with electrodes from the SevenMulti Modular Meter System (Mettler Toledo). Oxygen, alkalinity and chemical oxygen demand (COD) were analysed according to APHA (2005). Soluble reactive phosphorus (SRP), total phosphorus (TP), silicates (SiO_2) and chlorophyll *a* (Chl-a) were analysed spectrophotometrically using a Perkin Elmer Lambda UV-VIS spectrometer. Ammonium (NH_4^+-N), nitrites (NO_2^--N), nitrates (NO_3^--N) and chlorides (Cl-) were analysed using a Dionex 3000 ion chromatograph. Total nitrogen (TN) and total organic carbon (TOC) were analysed using a Shimadzu TOC-VCPH equipped with an analyser for TN and TOC.

A detailed description of the physical and chemical water analyses in Hungary can be found in Ács et al. (2025).

Light- and electron microscopic investigations

The Croatian quantitative phytoplankton samples were analysed using a Carl Zeiss Axio Observer inverted microscope with DIC according to the Utermöhl (1958) method (CEN 2006). A minimum of 400 settling units were counted in a transect at 400× magnification, resulting in a counting error of < 10% (Lund et al. 1958); CEN 2006). Larger cells and colonies were additionally counted at 200× and 100× magnification. Biovolume was calculated by determining the average size of an individual from up to 30 randomly selected cells of each taxon and then multiplied by the observed abundance of the species (Rott 1981). The biomass (fresh weight) was derived from the biovolumes and used for further analyses, where 1 mm^3 l^{-1} = 1 mg l^{-1} (CEN 2015a). The occurrence of *D. asterocostata* and its ecological significance are clarified by analysing its relative biomass within the phytoplankton community. The diatoms were identified using the permanent slides made by cleaning the net samples with warm hydrochloric acid and hydrogen peroxide and immersed in Naphrax solution (CEN 2009). They were identified under a microscope with 1000× magnification and differential interference contrast optics.

For scanning electron microscopy (SEM) of the Croatian samples, part of the cleaned suspension was filtered through polycarbonate membrane filters with a pore diameter of 2 µm, fixed on aluminium stubs after air drying and coated with 6 nm gold (Quorum Technologies, Q150R ES) at a deposition current of 120 mA for a period of 120 s. The SEM images were recorded with a field emission scanning electron microscope (FE-SEM, Tescan MIRA 3) at an acceleration voltage of 4 kV and a working distance of 5 mm. For the morphological studies of the Hungarian samples, the diatoms were cleaned with hydrogen peroxide and hydrochloric acid, washed with distilled water and filtered on to an Isopore polycarbonate membrane filter with a pore diameter of 3 µm (Merck Millipore), fixed to metal stubs with double-sided carbon tape, spot-painted with conductive silver paint (NO-VOC Silver Paint, SPI Supplies) and coated with gold using a Quorum Q 150R ES plus rotary-pumped spray coater. The prepared samples were examined at the HUN-REN Centre for Ecological Research in 2022 with a

Zeiss EVO MA 10 scanning electron microscope (SEM) at 10 kV and 10 mm working distance using a secondary electron (SE) detector. From each sample, 100 diatom valves belonging to the order Thalassiosirales were identified at a specific level. In this way, the count revealed the relative abundance of the species as a percentage. Further electron microscopic investigations were carried out in 2024 to produce a detailed morphological study of *Discostella asterocostata*. The investigations were carried out using a Zeiss Sigma 360 VP SEM operated at 5 kV and 6 mm working distance, also with an SE detector.

Preparation and pipeline of molecular analysis

DNA extraction was done from the ethanol preserved subsamples. Centrifuging was followed by lysis with AP1 buffer and incubation at 95 °C for 20 minutes. The process continued by Proteinase K digestion, RNase treatment and DNA purification was carried out according to the DNEasy Plant Mini Kit (Qiagen) protocol. Amplification of partial (263 bp) region of rbcL was executed by polymerase chain reaction (PCR) using primers from Vasselon et al. (2018) with Illumina P5/P7 overhang adapters. PCR products were purified with AMPure XP beads, quantified using a Qubit 4 Fluorometer, indexed using Nextera DNA CD Indices and quality-checked on an Agilent TapeStation 4150. The final library, diluted to 4 nM, was sequenced on the Illumina MiSeq platform (MiSeq V2(500) Reagent Kit and 2*250 bp read length). Library preparation and sequencing were performed by Biomi Ltd. (Hungary). Raw data were processed with the DADA2 pipeline (Callahan et al. 2016), which includes primer removal, quality filtering, read trimming, dereplication, merging of paired reads and chimera removal. The pipeline was further adapted to meet the specific requirements of diatom metabarcoding targeting the rbcL gene and has proven effective for this purpose (Keck et al. 2019). The Diat.barcode database v.11.1 (Rimet et al. 2019) was used for the taxonomic assignment, while the re-analysis was performed with the updated version 12.4. Sequence alignment was performed by Clustal W algorithm (Thompson et al. 1994) implemented in MEGA11 (Tamura et al. 2021). Phylogenetic tree construction and p-distance calculations were also carried out in MEGA 11. In particular, the Maximum Likelihood method was used to construct the tree, based on our newly-found *D. asterocostata* sequences, publicly available *Discostella* rbcL sequences, which are identified until species level and four diatom species (*Stephanodiscus niagarae*, *Cyclostephanos makarovae*, *Pantocsekiella ocellata* and *Lindavia radiosa*) used as outgroups, following a strategy similar to that of Schultz et al. (2024).

The Tamura 3-parameter model with a discrete gamma distribution was identified as the best-fitting substitution model by MEGA 11 and bootstrap values were evaluated through 500 replicates. The preparation protocols for both morphological and molecular analyses followed the methods described in Ács et al. (2025) and the references therein.

Data analysis

For the morphometric analysis of *D. asterocostata*, the entire permanent slide was scanned and all frustules present in the assemblages were measured. The morphometric analyses (also in the case of the Hungarian samples) were performed following Alverson et al. (2021), measuring eight morphological features

of the valve, including: five diameter measurements, count of the marginal strutted processes, the number of the total outer and total central striae. The results are compared with the morphometric data of *D. asterocostata* in China and in the U.S., given in Appendix S3 in Alverson et al. (2021). Principal component analysis (PCA) was performed for the comparison between the populations.

Spearman's correlation coefficient was used to examine the correlations between algal biomass and the environmental variables using IBM SPSS Statistics (IBM Corp. Released 2019).

Results

Environmental characteristics of the Mura, Drava and Danube Rivers

The physical and chemical properties of the Croatian rivers studied are summarised in Suppl. material 1, while the Hungarian data have already been published in Ács et al. (2025).

The water temperature ranged from 7.2 °C (Mura Goričan) to 28.1 °C (Danube Ilok) and showed an increasing trend downstream. The analysed rivers had a slightly alkaline character, with average pH values of 8.0 to 8.2. The dissolved oxygen concentration also followed a similar trend, with the lowest oxygen concentration measured in the Drava Botovo (8.6 mg l^{-1}) and the highest in the Danube Batina (9.7 mg l^{-1}). Alkalinity, conductivity and TSS also showed increasing trends downstream and peaked at the Danube Batina (145 mg $CaCO_3$ l^{-1}, 393 µS cm^{-1}, 22.0 mg l^{-1} TSS), with the exception of the Mura Goričan, which had the highest TSS concentration.

The average values of nutrients showed a decreasing trend for nitrogen compounds and dissolved silicates downstream from the Mura River to the Danube River, while phosphorus compounds showed a reverse trend and increased downstream. Total nitrogen ranged from 0.67 to 2.87 mg N l^{-1} and total phosphorus from 0.03 to 0.32 mg P l^{-1}.

Organic pollution in the form of BOD showed an increasing trend downstream, while COD, DOC and TOC were highest at the most upstream (Mura Goričan) and downstream (Danube Batina) sites. Chlorides followed a similar downstream increasing pattern, with the highest values at Danube Batina and Ilok. Chl-a concentrations increased downstream, reaching 79.7 µg l^{-1} and 65.7 µg l^{-1} at Danube Batina and Danube Ilok, respectively. The discharge ranged from 153.5 m^3 s^{-1} in the Mura Goričan to 1757.3 m^3 s^{-1} in the Danube Ilok with a water residence time of 14.3 to 63.4 days.

The data from Hungary fell into more or less similar ranges, with the exception of Chl-a, whose maximum value was significantly higher than the Croatian maximum value in both the Danube and the two tributaries analysed (in the Danube and the Mosoni Danube, the highest concentration was measured in March, while in the RSD, it peaked in September).

Phytoplankton composition with focus on *Discostella asterocostata*

Based on morphological observations, a total of nine major taxonomic groups (phyla) were identified from 123 samples from eight sites along the Mura, Drava and Danube Rivers: Bacillariophyta, Charophyta, Chlorophyta, Cryptophyta, Cyanobacteria, Euglenophyta, Miozoa, Ochrophyta and Rodophyta. Within

the phylum Bacillariophyta, 112 taxa were identified, belonging to the classes Bacillariophyceae (79), Coscinodiscophyceae (13) and Mediophyceae (20). The species *Discostella asterocostata* is categorised in the class Mediophyceae.

The total biomass of phytoplankton varied between 0.23 mg l^{-1} and 10.74 mg l^{-1} in the Danube Ilok in 2016 (August) and in the Danube Batina in 2017 (June). Bacillariophyta contributed the most to the total biomass compared to other phyla, accounting for between 44.6 and 99.1% of the total proportion of all samples. Of the classes Bacillariophyceae, Coscinodiscophyceae and Mediophyceae within the phylum Bacillariophyta, the total biomass is dominated by Mediophyceae (67.6%), while Bacillariophyceae and Coscinodiscophyceae account for 25.2 and 7.2%, respectively.

When analysing the oxidised samples, we identified *Discostella asterocostata*, a species that is new to both the Croatian and Hungarian flora. Although it had a very low proportion in the biomass of the samples (with the proportions in the Mediophyceae class, with the highest proportion of 3.2% recorded on the Drava before the confluence with the Danube in September 2017 and the lowest proportion (0.2%) in the Mediophyceae class on the Drava Belišće in May 2019 – Fig. 2), we started a detailed study of the species, as this had not been done before with any European population of the species.

Morphometric features of *Discostella asterocostata*

The valves are circular with strongly concentrically undulate valve face (Fig. 3). The valves have a diameter of 10 to 40 µm. The central area of the valve is strongly convex or concave (arrow 2) with radially arranged central striae. The valve margin has radial striae ranging from 11 to 16 in 10 µm. Under the light microscope, a clear ring-line can be observed in the mid-portion of the marginal striae (arrow 1). Long external elongate central tubes of marginal fultoportulae can be observed on scanning electron microscopy (arrow 3). The marginal fultoportulae, which can be seen as a ring of spots near the junction of the valve face and mantle (arrow 6), are located on every 2–4 striae. Internally, fultoportulae have 2 satellite pori (arrow 4). The central fultoportula is not present. A single rimoportula appears as a very small hollow on the margin of the valve; internally, it has a relatively well-developed labium, somewhat parallel (or oblique) to valve edge (arrow 5).

The cell size of the examined specimens from Croatia and Hungary ranges from 10.7 to 27.8 µm in diameter. The stria density in the observed valves ranges from 14 to 21 in 10 µm in the marginal area of the valve face.

Morphometric comparison to known populations from Asia and the U.S.

For PCA analysis, morphological characteristics of *D. asterocostata* were measured on 35 valves from Croatian and 10 valves from Hungarian samples and compared with morphometric data of *D. asterocostata* in China and the U.S., listed in Appendix S3 in Alverson et al. (2021). Principal component analysis showed that PC1 (for 91.8% of the variance in the dataset) was strongly correlated with cell diameter (Fig. 4A). Principal component axes 2 and 3 accounted for 6.6% of the variance and the populations of Croatia (Europe), Hungary (Europe), the U.S. and Asia completely overlapped on PC axes 2 and 3, suggesting that the four populations are indistinguishable from each other (Fig. 4B).

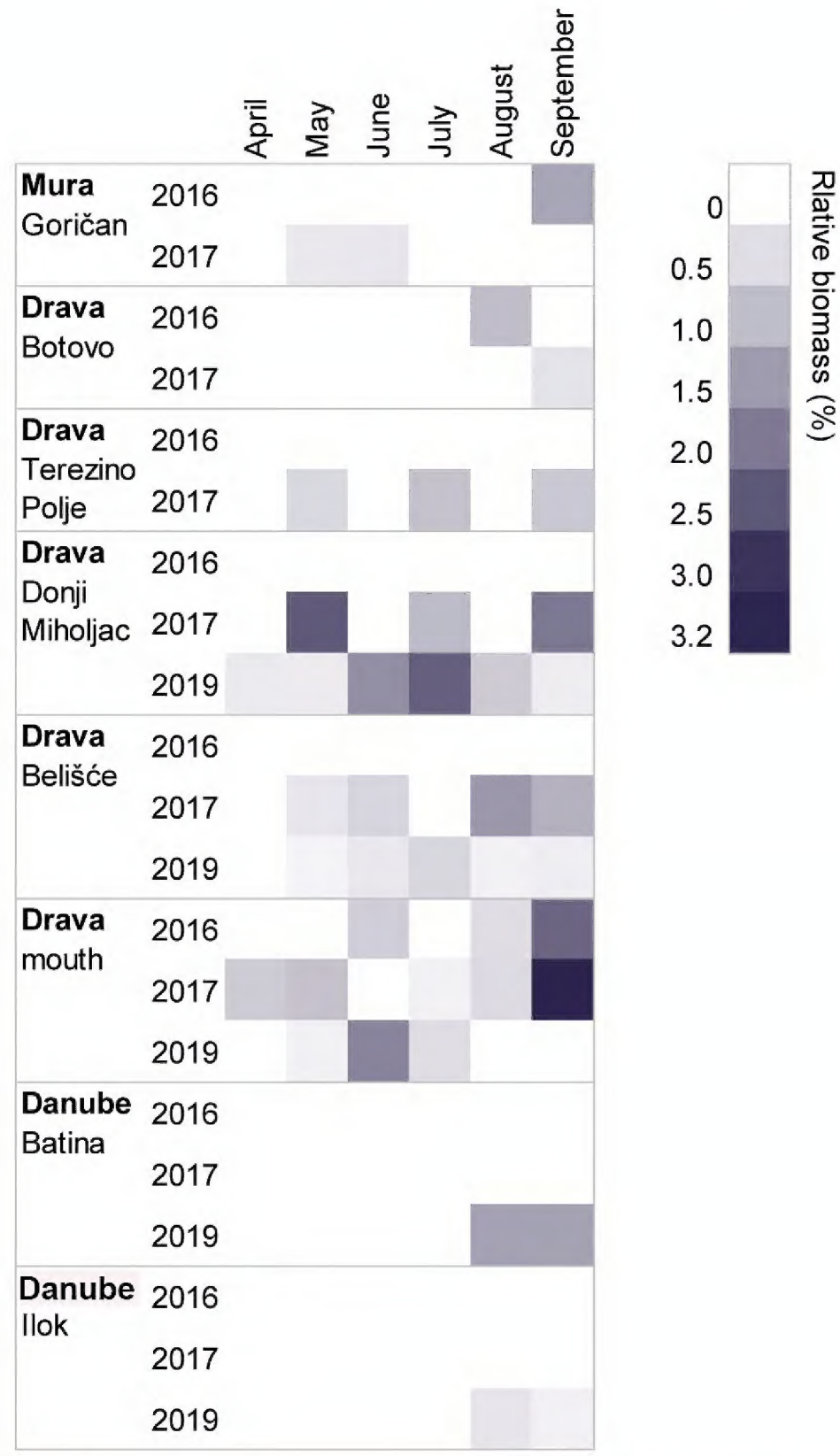

Figure 2. Relative biomass of *D. asterocostata* in the rivers Mura, Drava and Danube - based on quantitative microscopic analysis.

Ecology of *D. asterocostata*

To obtain a clearer picture of the ecology of *D. asterocostata* in the potamoplankton of very large rivers, the Spearman's rho coefficient was calculated in the case of the Croatian samples for all diatoms represented as the phylum Bacillariophyta, as three classes Bacillariophyceae, Coscinodiscophyceae and Mediophyceae and all taxa from the class Mediophyceae in which *D. asterocostata* is positioned with environmental variables.

Spearman correlation analysis of all environmental parameters showed a relationship between the environmental variables and all diatoms represented as phylum Bacillariophyta, three classes (Bacillariophyceae, Coscinodiscophyceae and Mediophyceae) and all taxa of class Mediophyceae, to which *D. asterocostata* belongs, represented by total biomass (Suppl. material 2). The species *D. asterocostata* favoured significantly warmer water in the summer months (positive correlation with water temperature) and water with less nutrients and organic pollution (negative correlation with nitrogen forms, TOC and chloride). A significant negative correlation was also found with discharge.

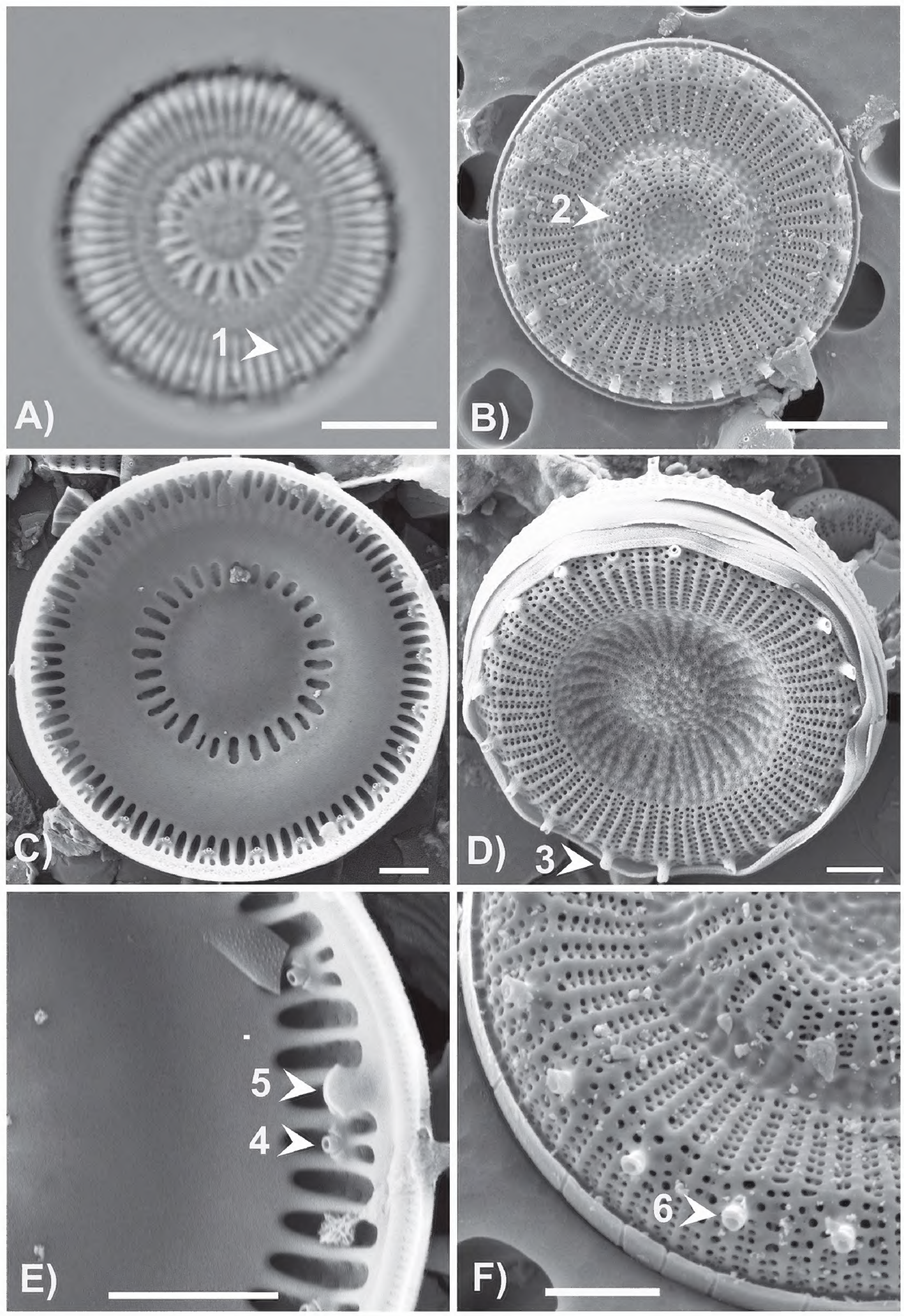

Figure 3. LM (**A**) and SEM (**B–F**) photomicrographs of *Discostella asterocostata*. **B.** External view of the valva; **C**. Internal view of the valva; **D.** External view of the frustulum with cingulum; **E.** Detail of the internal view; **F.** Detail of the external view. Arrow 1: Marginal striae with distinct shadow line; arrow 2: Valves strongly concentrically undulated; arrow 3: Marginal fultoportulae every 2–4 striae; arrow 4: Marginal fultoportulae (internally) with two satellite pores; arrow 5: Rimoportula with labium; arrow 6: Marginal fultoportulae (externally) with spine-like extensions. Scale bars: 5 µm (**A, B**); 2 µm (**C–F**).

In the case of the Hungarian samples, we calculated the Spearman correlation between the relative read number of *D. asterocostata* (based on molecular analysis) and the chemical variables (Suppl. material 3). The results confirmed the findings from Croatia and showed a significant positive correlation with temperature and a significant negative correlation with TOC and nitrogen forms (total nitrogen and nitrate).

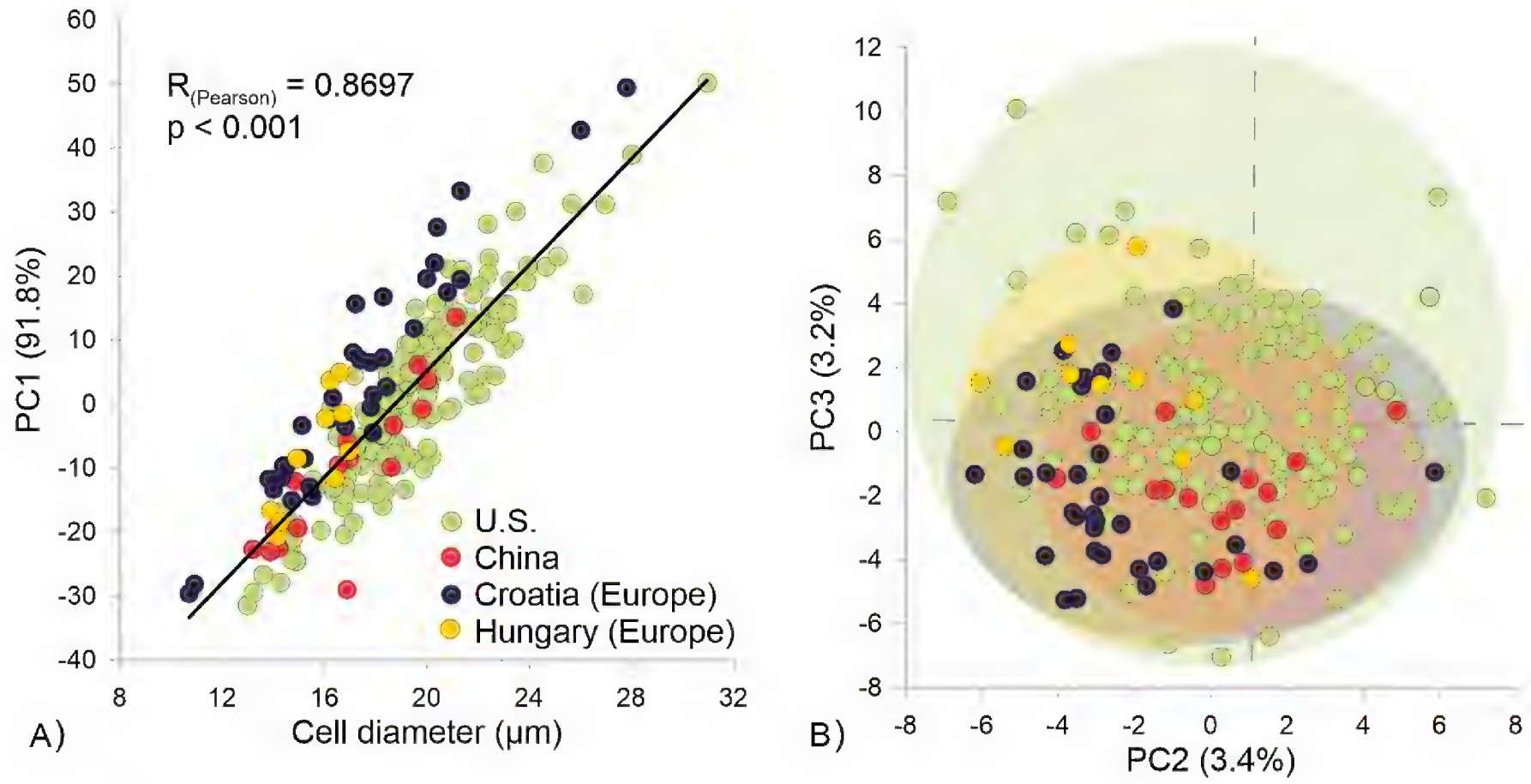

Figure 4. Principal component analysis (PCA) of eight morphometric characters for populations of *Discostella asterocostata* in Croatia (Europe), Hungary (Europe), the U.S. and China. **A.** Linear regression of the first PC axis against cell diameter; **B.** PCA plot comparing populations from Croatia (Europe), Hungary (Europe), the U.S. and China.

European occurrences of *D. asterocostata*, based on microscopic and molecular methods

D. asterocostata was observed in Croatian phytoplankton samples for the first time in 2016 during routine national biomonitoring. During the study period from 2016 to 2019, populations of *D. asterocostata* were found in Croatian samples from April to September at eight sites along the Mura, Drava and Danube Rivers and the first planktonic occurrence in Hungary was in 2021. In the first SEM survey, only a single valva of *Discostella asterocostata* was found in Hungarian RSD sample from July 2021 (although we counted 100–100 valves from each sample we collected at the 13 sites in March, May and July). We performed metabarcoding of all monthly collected Hungarian samples, but could not identify any *D. asterocostata* as it was missing in the Diat.barcode 11.1 version published on 25/05/2022. When we started working on the publication, we checked the latest version of the Diat.barcode database and found that, in 2023 and 2024, two research groups had published the first sequence of the rbcL region of *D. asterocostata* collected from three sites in China (the sequence under NCBI GenBank, Accession No. OQ029505.1 was directly submitted in 2022 by Xiang,Y., while OQ504849.1 and OQ504886.1 sequences were published by Schultz et al. (2024)). Of the three barcodes, OQ504849.1 and OQ504886.1 are identical, while OQ029505.1 is longer on both sides because it is from a genome sequencing that captured the entire rbcL gene rather than just one region. Based on this new information, we re-analysed our previously unclassified sequences, which had been identified to genus level as *Discostella*, but not assigned to any particular species. The uncorrected p-distance between our unidentified sequences and the recently published sequences was determined using MEGA 11 software. Three sequences (*Discostella_asterocostata_001*, *Discostella_asterocostata_002*, *Discostella_asterocostata_003* with the now available NCBI GeneBank accession numbers PV353726, PV353727 and PV353728, respectively) showed a high level of similarity (p distance < 0,01,

Suppl. material 4), with 0–1 base pair differences compared to the already published barcode. All three previously available (in NCBI GeneBank) sequences include the same rbcL barcode region as used in our study. This region is identical to *Discostella_asterocostata*_001, while *Discostella_asterocostata*_002 and *Discostella_asterocostata* _003 differ by one base pair each. After the new analysis, the species was found to occur in the entire Hungarian section of the Danube using molecular methods. However, it was found in higher abundance during the warm water period and was most abundant in the RSD (Fig. 5).

Since we carried out detailed light and electron microscopic studies of the phytobenthos of 328 samples from surface waters in Hungary in 2019–20 and also analysed the same samples molecularly by metabarcoding, we wondered whether the species might have been present in Hungary earlier. For this reason, we also re-analysed the unclassified *Discostella* sequences of these earlier samples, including the three known barcode sequences (one published, two from our samples). This led to the identification of two additional sequences: *Discostella_asterocostata_004* (NCBI GeneBank accession No. PV353729), which also differs by 1 base from the published barcode and *Discostella_asterocostata_005* (NCBI GeneBank accession No. PV353730) which differs by 2 bases compared to the published barcode, but only by 1 compared to *Discostella_asterocostata_002* (Fig. 6, Suppl. material 4). The results suggest that *D. asterocostata* was already present in Hungary in 2019, as it was detected at the following oxbow lakes Grébeci-Holt-Duna (floodplain), Bátai-holtág (floodplain) and Boki-holtág (protected site) in September 2020; Külső-Béda (floodplain) in September 2019 and Nagybaracskai-Holt-Duna (protected side) in September 2019. They are all located along the Danube in the southern part of Hungary, along the section of the Danube between Sükösd and Mohács (1492–1447 rkm). By 2021–22, the occurrence of the species has expanded to the entire Hungarian Danube (Table 1). Moreover, we re-analysed the unclassified *Discostella* sequences of phytobenthos samples collected in 2019 July during the 4th Joint Danube Survey and published by Tapolczai et al. (2024). We found that two of their unassigned *Discostella* ASVs (ASV_0703 and ASV_0813) are identical to our sequences *Discostella_asterocostata*_001 and *Discostella_asterocostata*_002, respectively. Notably, ASV_0813 was detected in the RSD site in Hungary, providing further evidence for the presence of *D. asterocostata* in the region in 2019.

Discussion

Although the biogeography of diatoms is an intensively researched field, it still raises unanswered questions. Some studies argue in favour of their cosmopolitan distribution, while others suggest that many species have a more restricted range (Williams 2025).

In contrast to earlier theories, such as the Baas-Becking (1934) hypothesis that microbes can disperse almost indefinitely across suitable habitats, microbial dispersal is not completely unlimited and human activities may play an important role in shaping biogeographical patterns (Alverson et al. 2021). Several studies have demonstrated dispersal limitations at different spatial scales and for different microorganisms (Custer et al. 2022; Barbour et al. 2023; Lemoine et al. 2023). The biogeographical distribution of certain microorganisms shows considerable variation and human activities can introduce them into new areas.

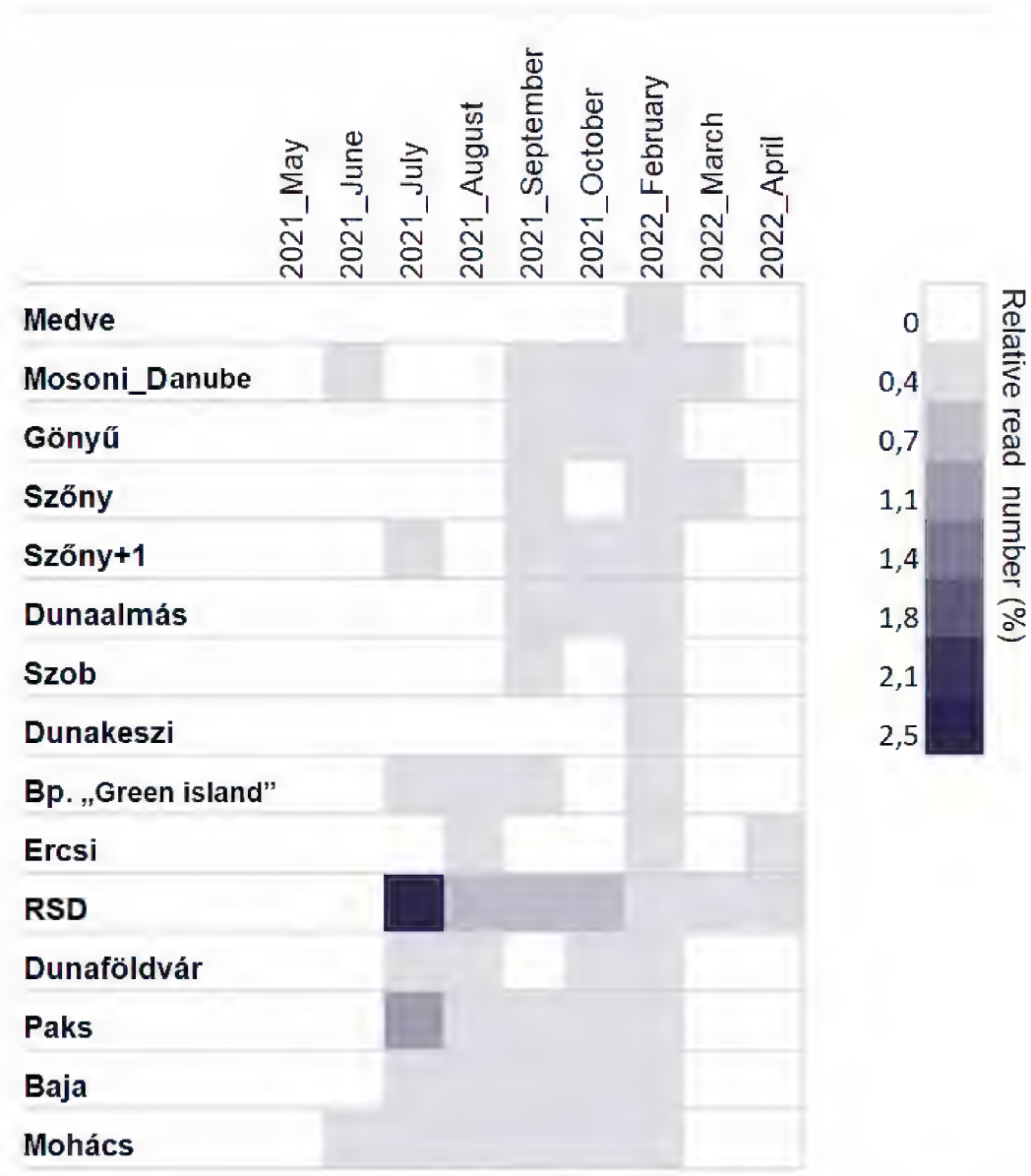

Figure 5. Relative read number of *D. asterocostata* in Danube samples - based on genetic results.

Table 1. Sequence variants of *Discostella asterocostata* at various sampling sites along the Danube and associated waters. An "x" indicates the presence of a particular variant at a particular location.

	Discostella_ asterocostata_001	***Discostella_ asterocostata_002***	***Discostella_ asterocostata_003***	***Discostella_ asterocostata_004***	***Discostella_ asterocostata_005***
Medve	x				
Mosoni Danube (Vének)	x				
Gönyű	x				
Szőny	x				
Szőny+1 km	x				
Dunaalmás	x				
Szob	x				
above Bp.	x	x			
Bp_"Green island"	x	x			
Ercsi	x	x			
RSD	x	x	x		
Dunaföldvár	x	x			
Paks	x	x			
Baja	x	x			
Mohács	x	x			
Grebeci-Holt-Duna	x	x			
Bátai-holtág	x	x			
Boki-holtág		x			
Külső-Béda		x			
Nagybaracskai-Holt-Duna	x	x		x	x

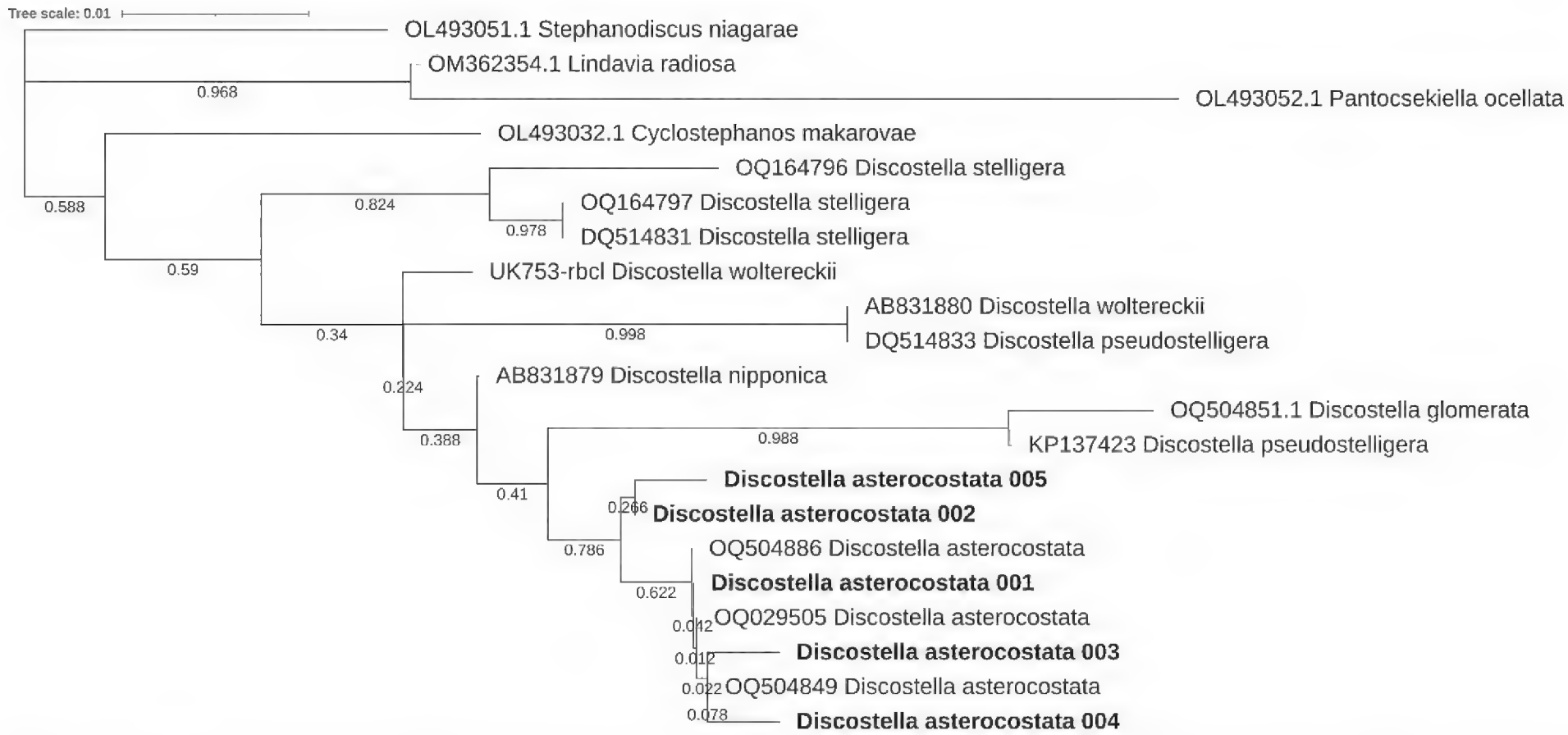

Figure 6. A Maximum Likelihood tree of the *D. asterocostata* sequences identified in this study, the published species-level identified *Discostella* sequences from the Diat.barcode database and *Stephanodiscus niagarae*, *Cyclostephanos makarovae*, *Pantocsekiella ocellata* and *Lindavia radiosa* sequences used as outgroups, constructed using the Tamura 3-parameter model. The sequences from the database are provided with the NCBI GenBank accession number. The tree is drawn to scale, with branch lengths representing substitutions per site. The analysis was performed in MEGA11. Node support was evaluated using 500 bootstrap replicates.

The species *Discostella asterocostata* was described in China in 1985 under the name *Cyclotella asterocostata* (Xie et al. 1985), but it was first observed during the scanning electron microscopy (SEM) examination of samples collected in the 1970s. Later, based on the position of the marginal fultoportula (between the costae), Houk and Klee (2004) transferred it to the genus *Discostella*.

In 2007, Tanaka (2007) reported its occurrence in Japan, while the species has also been recorded in South Korea (Lee et al. 1995; Houk et al. 2010) and in the eastern part of Russia (Medvedeva et al. 2009). A publication on its occurrence in North America appeared in 2021, but the species had already been detected in plankton samples from Beaver Lake, Arkansas (U.S.) and the Wabash River, Indiana (U.S.), in 2012 (Alverson et al. 2021).

Subsequent re-examinations of permanent slides from samples collected between 2015 and 2018 confirmed the presence of the species at a further 35 locations in the United States as well as at one location in the Asian part of Russia and three locations in South Korea. Sediment core analyses from several American lakes also revealed that the species has been present in North America since at least the mid–1980s (Alverson et al. 2021).

Despite the fact that freshwater centric diatoms have been intensively documented in Europe for decades (e.g. Houk et al. (2010); Kiss et al. (2012); Bey and Ector (2013); Ector et al. (2015); Peeters and Ector (2017), literature data as well as European monitoring programmes and our own collections suggest that the current (2016) occurrence of *D. asterocostata* is the earliest known record from Europe.

Prior to the present publication, there are only two earlier publications: *D. asterocostata* was found in the Sava and Tisza Rivers in Serbia (Tomović et

al. 2024), but they reported a much later occurrence (from 2021). Moreover, the species was found in the French and Belgian part of the Meuse River in 2023 and in 2024 (Heudre et al. 2024). As *D. asterocostata* has a very distinctive morphology, its previous absence in the European diatom records cannot simply be attributed to an observational error. Kiss has studied the phytoplankton of the Danube extensively for decades using light and electron microscopy (Kiss 1986; Kiss 1987; Abonyi et al. 2018; Abonyi et al. 2020; Rusanov et al. 2022). Between 2000 and 2004, detailed electron microscopic analyses of phytoplankton samples from 107 Hungarian rivers were carried out as part of a four-year sampling campaign (Kiss et al. 2012). In addition, the species was not morphologically detected in samples from the 2019 JDS4 survey along the Hungarian section of the Danube (Stanković et al. 2024).

We therefore believe that the detection of *D. asterocostata* in the Mura River in 2016 roughly coincides with its first appearance in Europe.

The species was first detected in the Danube in 2019 in Croatia (Stanković et al. 2024), as well as in Serbia (based on our re-analysis of benthic diatom sequences collected during the 4th Joint Danube Survey (JDS) and published by Tapolczai et al. (2024)). Interestingly, the species was also present in 2019 in a side-arm of the Danube River (Ráckeve-Soroksár Danube, also revealed by the re-analysis of JDS sequences); however, it was not detected at any site in the main arm of the Danube within Hungary, nor in any upstream sections or tributaries of the river. By 2021, the species was present throughout the entire Hungarian stretch of the Danube and was also detected in the Mosoni Danube, although only with a low relative read number.

The recorded occurrences of the species are shown in Fig. 7, but a further expansion is expected at least in Europe. Heudre et al. (2024) suggest that the species may be invasive; however, there is currently no supporting evidence, as it has not been recorded in high abundance at any site. Nonetheless, its rapid spread is indisputable.

Morphometric analyses, in particular the broad overlap along the PC2 and PC3 axes, indicate that the European populations do not differ morphologically from the Chinese and American populations. This suggests that populations of the species occurring on different continents have not diverged. This result is also supported by the minimal genetic differences between the Asian and European populations, although we were only able to compare the short rbcL region used for metabarcoding. The identified sequences of the species were either completely identical to or differed by only 1–1 nucleotide from the sequences published by Schultz et al. (2024), who found the species to belong to a clade well separated from other *Discostella* species. Our phylogenetic analysis also supports this result, as all *D. asterocostata* sequences, including both those identified in this study and the previously published ones form a monophyletic group distinct from other *Discostella* taxa (Fig. 6). Its most prominent morphological characteristic is considered to be the ring-shaped pattern in the central area, but the populations we examined also matched the other features they described (diameter: 10–40 µm, marginal fultoportulae (MFP) on every 3rd–4th striae, concave-convex valve surface, prominent complex tubes MFP, 11–16 striae in 10 µm in Schultz et al. (2024)).

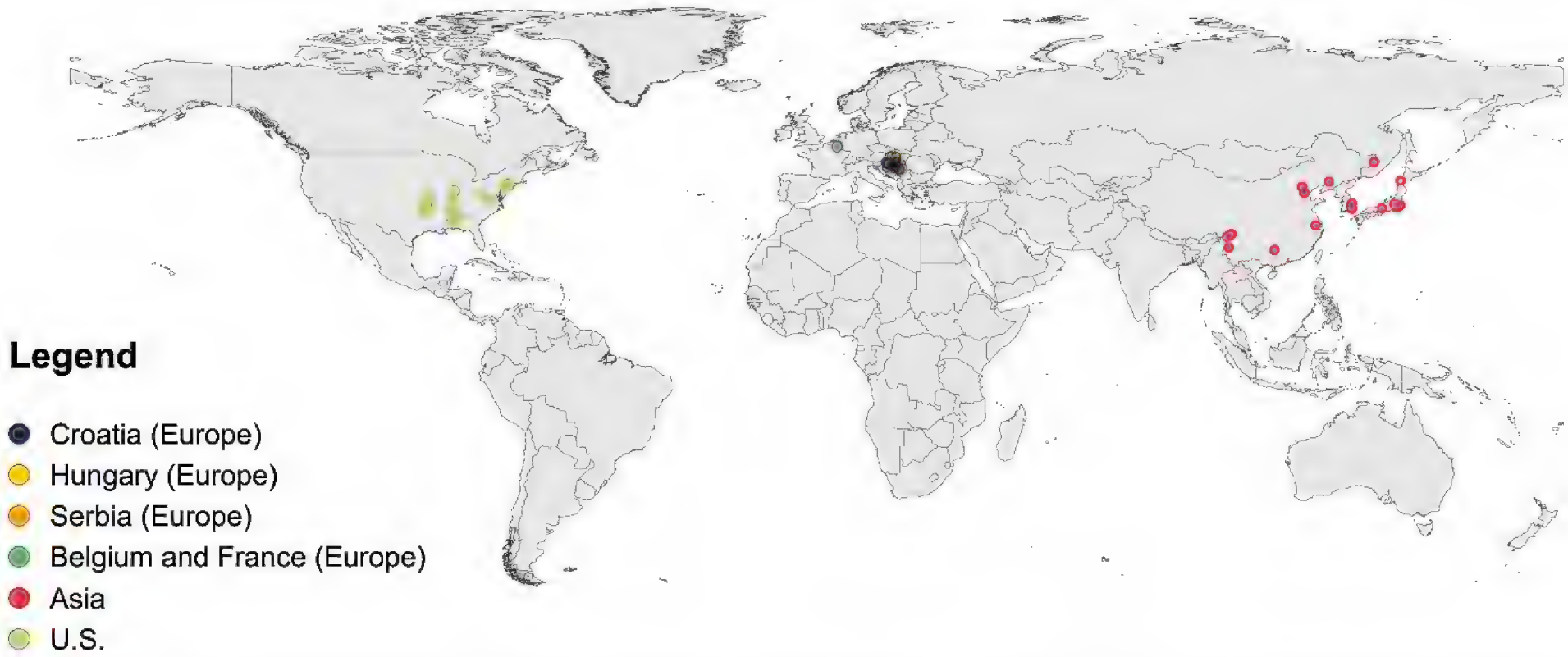

Figure 7. Global distribution of *Discostella asterocostata*. Records for U.S. and Asia are given in Alverson et al. (2021).

The 1–2 nucleotide differences observed in the rbcL region ASVs are most likely an indication of intraspecific variation (Vanormelingen et al. 2013) and this variation might be greater in populations that have been present in the waterbody for a "longer" period of time (e.g. in the RSD, where the species has been present since 2019 and three variants of the examined rbcL region have been identified). The other possible explanation for a few base pair differences between the barcode sequences is the possibility of technical errors. Nevertheless, there are several factors that reduce the likelihood of such problems, including strict quality control followed by the removal of low-quality reads and sections. The sequences were identified in several independent reads, which suggests that there are no such errors. Finally, the occurrence of these sequences at different sampling sites suggests a biological rather than a technical origin.

The strong positive correlation between cell diameter and the first principal component (PC1) shows that morphological variability increases with cell size. This is a common trend in this type of dataset and the same result was obtained by Alverson et al. (2021) when comparing Chinese and American *Discostella asterocostata* populations. When studying a centric diatom population with a high number of individuals, it is often observed that smaller individuals show minimal patterning compared to their larger cells (Kiss et al. 2002; Genkal and Popovskaya 2008).

In the case of the species appearance in the U.S., one possible vector identified is invasive Asian carp (silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*)), as their habitat overlap and feeding habits may have contributed to the diatom's introduction to North America (Alverson et al. 2021). However, considering that these two cyprinid fish species and the grass carp (*Ctenopharyngodon idella*) were introduced to Hungary from East Asia as early as 1963 (Takács et al. 2017), it is unlikely that they also explain the appearance of the species in Hungary.

The spread of algae can occur via various vectors, for example, ship ballast water, bird feet and faeces, wind etc. (Proctor 1959; Hallegraeff

1998; Tesson et al. 2016). However, for a species to establish itself as a stable member of an ecosystem, the environmental conditions must also be suitable. The introduction to Europe could have occurred through multiple pathways, including fish stocking or transport via ballast water from ships. However, the fact that *D. asterocostata* rapidly appeared from Croatia in oxbow lakes in southern Hungary – both in the floodplains and in protected oxbow lakes – and in RSD before it was even discovered in the main channel of the Danube in Hungary, suggests that waterbirds may have played a role as dispersal vectors (Lovas-Kiss et al. 2019).

Nevertheless, the potential vector role of Asian carps (*Hypophthalmichthys molitrix* and *H. nobilis*) cannot be excluded either. In particular, the silver carp (*H. molitrix*), which primarily feeds on floating algal biomass filtered from the water using the filtering apparatus on its gills, may play a key role. Silver carp larvae larger than 15 mm in length switch from zooplankton to phytoplankton feeding. Adults are capable of filtering particles and algae larger than 10 µm from the water. Given the sometimes limited digestive efficiency, some algae may remain undigested in the gut and be excreted alive in the faeces (Lovas-Kiss et al. 2019). This makes silver carp a potentially effective vector for algal dispersal within a waterbody. For dispersal between waterbodies, fish-transport water containing *D. asterocostata* may serve as the transmission medium.

Although Hungarian law prohibits the stocking of silver carp in natural waterbodies, including the RSD, it cannot be ruled out that silver carp were unintentionally introduced as a vector of *D. asterocostata* — possibly as a contaminant in the 180,000 kg/year of common carp (*Cyprinus carpio*) stocked in the RSD. Additionally, the possibility that this species was introduced via the water used to transport stocked fish also cannot be dismissed.

At the same time, it is also true that the species was not found microscopically either during the 2019–20 Hungarian phytobenthos surveys or in phytobenthos samples of 4th JDS. However, as *D. asterocostata* is a planktonic species, it is possible that it was present in such small quantities in the phytobenthos that it did not show up in the routine counts (where typically 400–500 valves are counted), while it was detected in the much more sensitive DNA analysis. It is also possible that only DNA traces of the species were detected in the oxbow lakes (as read number was consistently very low), especially as diatoms can remain viable after digestion (as mentioned above). Nonetheless, while eDNA is an extremely promising and effective tool for monitoring and early detection of invasive species (Morisette et al. 2021), accurate interpretation of results can be challenging due to issues such as the exclusion of false positive samples (Rishan et al. 2023). However, the fact that the species was in 2021 also identified microscopically in the RSD and has since been detected there every year (own observation) rather supports the interpretation that eDNA-based methods are more sensitive tools for the early detection of species.

Discostella asterocostata was never the dominant centric diatom in phytoplankton samples, but since its appearance in 2016, it can be said that the species has become a permanent member of the phytoplankton at its occurrence sites in Croatia (own observation). The negative correlation with nutrients indicates that it prefers water with a lower organic load. It favours

warm water periods (strong positive correlation with water temperature) and slow-flowing or stagnant waters (negative correlation with flow (Q)). The ecological preferences of *D. asterocostata* differ from those of the dominant centric diatoms, as shown by the positive correlation with Chl-a (*Cyclostephanos dubius* (Fricke) Round, *Cyclostephanos invisitatus* (Hohn & Hellermann) Theriot, Stoermer & Håkasson, *Discostella pseudostelligera* (Hustedt) Houk & Klee, *Skeletonema potamos* (C.I.Weber) Hasle, *Stephanocyclus meneghinianus* (Kützing) Kulikovskiy, Genkal & Kociolek, *Stephanodiscus hantzschii* Grunow and *Stephanodiscus minutulus* (Kützing) Cleve & Möller), which suggests that very large European rivers (Stanković et al. 2024) do not currently provide an optimal habitat for the expression of its invasive character.

The occurrence of this species in Europe is a good example of the accelerated changes in species composition worldwide. These changes also affect the interactions between species in aquatic ecosystems, which could jeopardise the functioning and stability of the entire ecosystem in the long term.

Due to international shipping and other vectors detailed above, more and more non-indigenous species are appearing in new areas. This makes NGS a crucial tool to quickly and accurately identify alien species and track their dispersal patterns, even at low population densities. In nature conservation, this can facilitate the development of early warning systems and reduce the harmful effects of invasive species. The technology also enables the creation of long-term databases that contribute to the understanding and monitoring of ecosystem changes.

Conclusions

The preference of *Discostella asterocostata* (the first documented example of which we report here in Europe's second largest river, the Danube and the earliest occurrence in European freshwater ecosystems) for warm, nutrient-poor and slow-flowing waters, together with its expansion pattern, support the hypothesis that human activities may have facilitated its spread. Its clear temperature-related seasonal dynamics, particularly during the summer months, suggest that climate change-induced warming of freshwater systems could promote its further dispersal and increased abundance. While *D. asterocostata* currently does not demonstrate invasive behaviour in European rivers, its presence exemplifies how rising water temperatures and enhanced dispersal pathways can contribute to the ongoing reshaping of aquatic ecosystems. The employment of molecular tools, such as metabarcoding, has been instrumental in the detection and monitoring of this species, particularly in contexts where it is present in low numbers. Consequently, the ongoing integration of high-throughput molecular techniques within routine monitoring frameworks is recommended, with a view to enhancing the early detection of non-native species and assessing their ecological implications under changing environmental conditions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Minimum, maximum and average values of physical, chemical and hydrological parameters and chlorophyll *a* for all sampling sites for the period April to September from 2016 to 2019

Authors: Igor Stanković, Sára Shemesh, Nikola Hanžek, Mirela Šušnjara, Marija Gligora Udovič, Keve T. Kiss, Zsuzsa Trábert, Zsolt Udvari, Tibor Bíró, Edit Vadkerti, Éva Ács

Data type: xlsx

Explanation note: (Croatian data only).

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Link: https://doi.org/10.3897/mbmg.9.154667.suppl1

Supplementary material 2

Spearman's rho correlations (2-tailed) between taxa and environmental parameters

Authors: Igor Stanković, Sára Shemesh, Nikola Hanžek, Mirela Šušnjara, Marija Gligora Udovič, Keve T. Kiss, Zsuzsa Trábert, Zsolt Udvari, Tibor Bíró, Edit Vadkerti, Éva Ács

Data type: xlsx

Explanation note: * Correlation is significant at the level $p \leq 0.05$; ** Correlation is significant at the level $p \leq 0.01$ in bold; n.s.: not significant; total number of samples = 126 (Croatian data only). The codes of the environmental parameters can be found in Suppl. material 1.

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Link: https://doi.org/10.3897/mbmg.9.154667.suppl2

Supplementary material 3

Spearman's rho correlations between the relative read number of *D. asterocostata* in Danube samples and the physicochemical variables of the samplings

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Data type: xlsx

Explanation note: * Correlation is significant at the level $p \leq 0.05$; ** Correlation is significant at the level $p \leq 0.01$; total number of samples = 43 (Hungarian data only).

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Link: https://doi.org/10.3897/mbmg.9.154667.suppl3

Supplementary material 4

Uncorrected p-distance values between the sequences of *D. asterocostata* identified in this study and published sequences from the Diat.barcode database

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Data type: xlsx

Explanation note: The sequences from the database are provided with the NCBI GenBank accession number. The sequences were trimmed to the same length, so that a total of 263 nucleotide positions are contained in the dataset.

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